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PRINCIPAL INVESTIGATOR: David Malkin

CONTRACTING ORGANIZATION: The Hospital for Sick Children
Toronto, Ontario Canada M5G 1X8

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Key Research Accomplishments from Dr. Malkin toward Award # W81XWH_10-1-0672_CA093469: Molecular Targeted Therapies of Childhood Choroid Plexus Carcinoma (CPC)

INTRODUCTION

Choroid plexus carcinoma (CPC) is a rare malignant brain tumor originating from the epithelial cells lining the cerebral ventricles. CPC represents less than 0.6% of brain tumors in all age groups, yet is more frequent in children (2-4%), especially in infants under the age of 1, accounting for over 20% of brain tumors in this age group (1). The molecular events that drive the malignant progression of this tumor are not well understood, yet this knowledge is crucial to improve patient survival. Surgical resection combined with neo-adjuvant and/or adjuvant therapy remain the primary methods of treatment for CPC; however tumor progression and relapse is observed in ~70% of cases (2). Despite improvements on the most current treatment protocols, long-term survival of CPC patients remains under 30% and survivors display significant neurocognitive and/or sensory deficits. (2,3). Identifying altered genes that drive the progression of CPC will refine current diagnostic and prognostic classifications of CPC patients, and promote the implementation of targeted therapies to improve patient survival and reduce long-term side effects. The proposed research aims to identify genetic lesions involved in CPC tumorigenesis in order to implement their use as unique markers for diagnostic and prognostic classification of choroid plexus tumor patients, as well as to promote the creation of personalized molecular targeted therapies.

I hypothesize that recurrent genetic lesions accompanied by a significant change in gene expression in CPC, will be drivers of tumorigenesis in this malignant brain tumor. Consequently, this research project will seek to answer the following question: What genes may be used as molecular markers for diagnostic and prognostic classification of CPC, and for which tumor-promoting alterations could CPC therapies be created?

BODY AND KEY RESEARCH ACCOMPLISHMENTS

We have made considerable progress in the second 12 months of our project; completing all the elements proposed in our Statement of Work (SOW). We have maintained twice monthly webinars and data sharing exchanges with the group at St. Jude throughout the last funding period. These have proven extremely important, ensuring that we exchange data and ideas in real time, maximizing the efficiency of all project interactions. The following narrative is set in the context of our group SOW, highlighting specifically those tasks assigned to Dr. Malkin. Sections of the original SOW are shown in italics. Details of tasks completed in year 1 follow in Roman text and details of tasks completed in **year 2** are in **bold, underlined text**.

SOW Specific Aim 1, Task 1

Specific Aim 1: To identify candidate drug targets of CPC.

Task 1: Generation of additional human and mouse CPC genomic profiles (timeframe: months 1-5).

The goal of these studies is to expand our number of genomic profiles (DNA and mRNA arrays) of both human and mouse CPCs to provide a comprehensive dataset with which to identify key candidate oncogenes, tumor suppressor genes (TSGs) and aberrant signal pathways that underlie the development of CPC. NB. Dr. Malkin will continue to generate human genomic profiles and compare these to mouse CPC profiles throughout the duration of the grant.

Task 1.d.: Identify and classify each of the copy number alterations (CNAs) in initial cohorts of human and mouse tumors as 'frequent' (>20% of tumor samples), moderately frequent (>10% to <20%), or rare (<10%) and according to distribution between human TP53 wild-type or mutant tumors and among the different stages of mouse tumor development. PIs: Gilbertson, Malkin, Guy and Ellison. Timeframe: months 4-5.

Task 1.e: *Accrue and perform central histology review and Affymetrix 6.0 SNP and Exon expression arrays of an additional human CPCs during the course of the granting period. PIs: Malkin, Ellison, Gilbertson. Timeframe: months 1-36 (ongoing throughout grant period).*

Milestone #1: *Completion of initial human and mouse CPC genomic datasets.*

Work completed toward Specific Aim 1, Task 1 by Dr. Malkin

Generation of additional human CPC genomic datasets

- DNA and RNA extraction was conducted for more than 90 human choroid plexus samples using well-established protocols.
- A variety of quality control tests were run to assess the integrity and quality of all samples. Fifty-nine DNA samples were selected for hybridization to Affymetrix SNP 6.0 GW microarrays, while 40 RNA samples were selected for reverse transcription reactions yielding cDNA for hybridization to Affymetrix Exon 1.0 ST microarrays.
- SNP and Exon microarrays were run at the Toronto Centre for Applied Genomics (TCAG) microarray facility.

This represents a significant contribution in the form of additional new human tumor data toward the analysis of CPCs. These data have been used directly to complete Task 2.

SOW Specific Aim 1, Task 2

Task 2: *Genome-wide cataloging of candidate oncogenes and TSGs in human and mouse CPCs (Timeframe: months 5-8).*

The goal of these studies is to identify candidate oncogenes and TSG that map within the CNAs detected in human and mouse CPCs.

Task 2.a: *Catalog all genes located within the frequent, moderately frequent and rare CNAs identified in human and mouse CPCs. PIs: Gilbertson and Malkin. Timeframe: months 5-6.*

Task 2.b: *Rank probability of the human and mouse genes within all inferred CNAs as being candidate oncogenes or TSGs of CPC. PIs: Gilbertson and Malkin. Timeframe: months 5-6.*

Task 2.c: *Select the top 30 candidate oncogenes and TSGs of human and mouse CPCs. PIs: Gilbertson, Malkin, Ellison and Guy. Timeframe: month 6.*

Task 2.d: *Real-Time PCR and FISH validation of copy number alterations of our lead candidate oncogenes and TSGs in human and mouse CPCs. PI: Ellison. Timeframe: months 6-8.*

Milestone #2: *Selection of the top candidate oncogenes and tumor suppressor genes of CPC.*

Work completed toward Specific Aim 1, Task 2 by Dr. Malkin

Genomic and transcriptomic analyses to identify candidate tumor suppressor genes and oncogenes

All raw data generated for each DNA and mRNA tumor microarray was used for copy number and gene expression analysis. These are now all complete and cataloged for alterations as detailed below.

Copy Number Analysis-Task 2a.

- Copy number analysis was performed using Partek Genomics Suite (PGS, Partek Inc. St. Louis, MO) and visualized using Integrative Genomic Viewer (IGV, <http://www.broad.mit.edu/igv/>)
- Normalization of all data was then performed with SNP 6.0 files created for the third phase of the International HapMap Project (HapMap3, 1301 control samples)
- We identified all areas of copy number alteration (CNA) that might contain oncogenes and tumor suppressor genes using the Hidden Markov Model (HMM) and Partek's segmentation algorithm. The minimum number of markers per region was set at 10.
- Candidate CNAs were statistically reviewed by a rigorous set of criteria to prioritize regions of gain or loss for further analysis.

Gene Expression Analysis-Task 2a and b.

- Gene expression analysis was performed using PGS and Gene Pattern (<http://genepattern.broad.mit.edu/>).
- Four clustering algorithms (principal component analysis (PCA), unsupervised hierarchical clustering (UHC), non-negative matrix factorization (NMF) and Consensus Clustering (CC)), were used to identify tumor subgroups that segregate independently according to their expression profile. In all analyses, a significant segregation was observed between CPC and choroid plexus papilloma (CPP) samples.
- Microarray intensities were analyzed in PGS, using the benign human choroid plexus papilloma (CPP) samples as an expression baseline reference. This analysis highlights expression changes unique to the malignant choroid plexus carcinoma (CPC) phenotype. Significant changes in expression between CPC and CPP were considered as a fold-change of 2 or more. P-value FDR= 0.05 after ANOVA analysis

Copy number-driven expression-Task 2b and c

- To analyze copy number-driven expression in choroid plexus tumors, we merged the copy number for each segmented region obtained from copy number analysis to the gene expression values.
- The data was filtered according to direction of copy number alteration and expression changes. Only those regions with copy number gain and increased expression, as well as copy number loss and decreased expression were selected.
- Further data filtering took into consideration the frequency of copy number-driven events in the CPC group compared to the CPP group, as the focus was on CPC-unique events.
- An initial list of recurrent CPC-unique regions with significant copy number-driven expression was compiled. These regions were observed in at least 56% of CPC samples (n=10/18). Seventy seven CNAs containing candidate oncogenes were identified. Most amplifications map to chromosome 1, but also include on chromosomes 5, 7, 8, 9 12, 14, 15, 17, 18, 19, 20, 21, X. (**Appendix 1,2**)

Validation of regions of copy number gain with FISH

- **FISH was used to validate the copy number gain observed in the catalog of aberrant genes in human chromosome 1.**
- **Probes mapping to human chromosome region 1p34 and 1q21 were created and utilized in fresh frozen paraffin-embedded human CPC.**
- **A small independent cohort of four CPCs was tested and two samples exhibited recurrent copy number gain of both regions, indicating a gain of the entire chromosome 1. Up to 4 copies of each region were observed.**
- **Further FISH analyses will be performed with a larger independent cohort.**

All data were discussed in real time with the entire group to ensure that we prioritized and selected the appropriate lesions for further analysis (See the reports of Drs. Ellison and Gilbertson in particular). Regions prioritized as special interest in cross species analyses as likely to contain oncogenes of mouse and human CPCs were then passed forward to Task 3.

Together with Drs. Gilbertson and Ellison we have been working to validate in human CPCs a series of alterations that are also observed in mouse tumors. These CNAs that are common to both species are likely to contain critical genes important in the development of these tumors.

Validating regions with qPCR and FISH-Task 2d.

- Validation of genomic and transcriptomic findings in human CPC by qPCR and FISH are currently in progress. Preliminary qPCR results have confirmed copy number gain in at least four CPC samples for the following genes: STMN1 (1p36), EXO1 (1q43), STIL (1p32), ZYG11A (1p32), RAD54L (1p32), and SLC1A7 (1p32).

Validation of regions of overexpression with Real-time PCR

- Real-time PCR was used as an orthogonal approach to validate the overexpression observed in the catalog of aberrant genes in human CPCs
- Human normal total brain RNA was used as the control sample, and *ACTB1* (Actin beta: 7p22) and *ALAS1* (aminolevulinate, delta-, synthase 1: 3p21) as reference genes.
- The overexpression of the following genes in CPCs have been validated:
 - *RAD54L*
 - *ATP6V0B*
 - *DVL1*
 - *GPATCH3*
 - *USP1*
 - *RPA2*
- Further real-time PCR analyses are currently underway.

Allele specific copy number analysis of CPCs

- The highly aberrant genetic profile of CPCs was dissected using a popular software program (ASCAT, Van Loo 2010, PNAS) that identifies copy number changes using SNP microarrays, taking into consideration tumor ploidy and sample heterogeneity.
- ASCAT confirmed previous observations, in that copy number gain of chromosome 1 was identified in more than 63% of the CPCs analyzed, despite the high frequency of tumor aneuploidy in CPCs.
- A subset of CPCs (43%) exhibited a hyperdiploid genome with 3 or more copies of chromosome 1, while another subset of CPCs (43%) exhibited a hypodiploid genome and most of these samples retain 2 copies of chromosome 1.

Pathway analysis of CPCs

- Gene set enrichment analyses were conducted on the original set of CPC for which exon microarrays were created in order to identify biological functions and pathways for which differentially expressed genes are enriched.
- The publicly available GSEA software program (Broad Institute, Cambridge MA), and a defined list of curated pathway gene sets from various online databases were utilized.
- Dataset permutations were conducted (n=1000), comparing enrichment in CPCs and the benign choroid plexus papillomas.
- The most significant datasets enriched in CPCs were:
 - Mitosis (normalized enrichment score=2.61, FDR p<0.001)
 - Spindle organization (normalized enrichment score=2.48, FDR p<0.001)
 - Kinetochore (normalized enrichment score=2.36, FDR p<0.001)
 - *FOXMI* transcription factor pathway (normalized enrichment score=2.32, FDR p<0.001)
 - Chromosome segregation (normalized enrichment score=2.25, FDR p<0.001)
- NB. Drs. Gilbertson and Ellison have also confirmed CNAs in these same genes in mouse CPCs. These are extremely exciting data since they identify for the first time candidate oncogenes of CPCs that are present in tumors in two separate species. We are now working with Dr. Guy to determine if these genes are targetable with novel therapies.

SOW Specific Aim 1, Task 3

Task 3: Interspecies analysis of gene candidates and pathway alterations common to human and mouse CPCs (Timeframe: months 6-8).

The goal of this analysis is to identify a set of common genes that are dysregulated in both human and mouse CPCs. Genes that are non-redundant for CPC tumorigenesis across species are likely to be critical to the disease process.

Task 3.a: Compare lists of orthologs of candidate oncogenes and TSGs between mouse and human CPC. **PIs:** Gilbertson and Malkin. **Timeframe:** months 6-8.

Milestone #3: Comprehensive understanding of the molecular similarities between human and mouse CPC.

Work completed toward Specific Aim 1, Task 3 by Dr. Malkin

Comparison between mouse and human data-Task 3a.

- The list generated from regions exhibiting copy number-driven expression in human CPC was used to compare equivalent regions in mouse CPC. The cross-species analysis was analyzed by the Gilbertson group.

REPORTABLE OUTCOMES

ABSTRACTS:

1. Merino D, Shlien A, Pienkowska M, Tabori U, **Malkin D**. *Aberrant copy number driven expression in chromosome 1 is involved in choroid plexus carcinoma progression*. EMBL Genome Workshop. Heidelberg, Germany. October 4-8, 2011.
2. Merino D, Pienkowska M, Shlien A, Malkin D. *Aberrant copy number-driven expression in chromosome 1 is involved in choroid plexus carcinoma development*. 2011 SickKids Research Institute Retreat. Toronto, ON. November 17, 2011.
3. Merino D, Pienkowska M, Shlien A, Malkin D. *Aberrant copy number-driven expression in chromosome 1 is involved in choroid plexus carcinoma development*. Annual Meeting, American Association for Cancer Research. Chicago, IL. April 1-4, 2012.
4. Merino D, Pienkowska M, Shlien A, Tabori U, Gilbertson R, Malkin D. *Mutations in p53 distinguish two molecular subgroups in choroid plexus tumors*. Canadian Student Health Research Forum (CIHR) Winnipeg, MB. June 13, 2012. [platform presentation]
5. Merino D, Pienkowska M, Shlien A, Tabori U, Gilbertson R, Malkin D. *Mutations in p53 distinguish two molecular subgroups in choroid plexus tumors*. International Society of Pediatric Neurooncology. Toronto, ON. June 23-27, 2012. [platform presentation]

CONCLUSIONS

Our data to date indicates differential patterns of gene amplification and deletion between choroid plexus papilloma and malignant choroid plexus carcinoma. The most striking chromosomal targets are throughout chromosome 1, and these appear to correlate highly with mouse orthologs. This data provides a robust basis on which to pursue functional characterization, cellular localization and drug target discovery studies in the next 2 years of the grant cycle.

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3. Lafay-Cousin, L. et al. (2010) Choroid plexus tumors in children less than 36 months: the Canadian Pediatric Brain Tumor Consortium (CPBTC) experience. Childs Nerv Syst 27(2), 259-264
4. Lafay-Cousin, L. et al. (2010) Use of ifosfamide, carboplatin, and etoposide chemotherapy in choroid plexus carcinoma. J Neurosurg Pediatrics 5(6), 615-621

APPENDIX 1 (Figure 1)

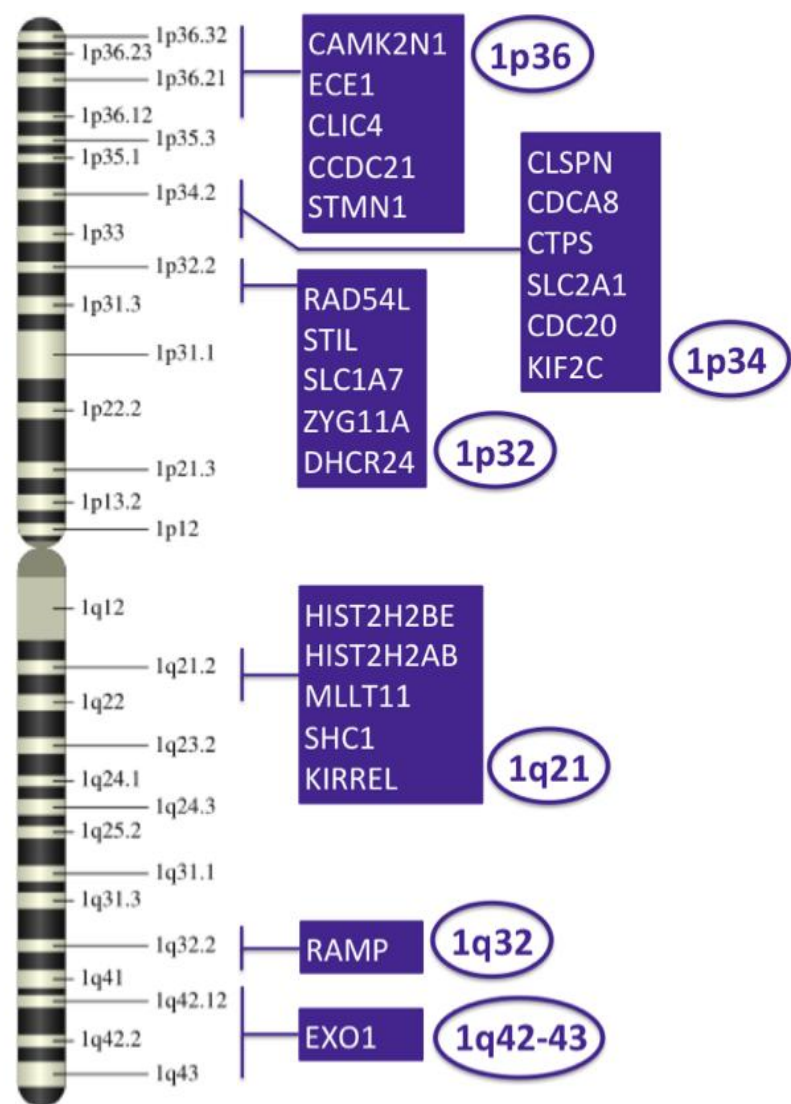


Figure 1: Chromosome 1 contains 23 significant regions of copy number-driven expression unique to CPC.

Appendix 2 (Figure 2)

Figure 2: Profile of chromosome-wide CNA in CPP and CPC samples. Black squares represent regions of copy number gain (≥ 2.75) or loss (≤ 1.25) that cover more than 50% of each chromosome arm. P-values calculated by Fisher's exact test (significant p-values in red).

[illegible]